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MASS SPECTROMETRIC IDENTIFICATION OF THE RADICAL ADDUCTS OF A FLUORESCAMINE-DERIVATIZED NITROXIDE

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The radical adducts of fluorescamine-derivatized 3-aminomethyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical are readily identified by mass spectrometry employing a solid source probe and electron impact ionization.

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INTRODUCTION

In two previous papers,[1,2] we described a simple method to detect very low levels of carbon-centered radicals in aqueous systems. The method employs a water-soluble amino nitroxide (3-aminomethyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical, 3-AMP) to efficiently trap carbon-centered radicals as stable alkoxyamine adducts. Derivatization of these adducts with fluorescamine produces highly fluorescent compounds, while derivatization of 3-AMP by itself gives rise to a substantially less fluorescent compound owing to efficient intramolecular quenching by the nitroxide.[3-5] These compounds are easily separated by reversed-phase high performance liquid chromatography (HPLC) and are detected fluorimetrically.

Previously, the identities of the adducts were assigned indirectly through comparison of the elution patterns obtained for the products of photochemical reactions producing known radicals.[1,2] Here we show that these adducts can be unambiguously identified by mass spectrometry (MS) employing a solid source probe and electron impact ionization. While MS has been used in earlier studies to identify the radical adducts of spin traps, (see for example, ref. 6) and the adducts produced by nitroxide scavenging of radicals[7,8] this work demonstrates that MS can also be employed for the structural identification of the radical adducts of the nitroxide-fluorophore probes.[1-5]

MATERIALS AND METHODS

Chemicals

Boric acid, ketones and 3-AMP were purchased from Aldrich (U.S.A.), while the α-keto acids and fluorescamine were obtained from Sigma (U.S.A.). Ketones, α-keto

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acids and fluorescamine were used as received; 3-AMP was purified as previously described.[2] Distilled-in-glass grade solvents were from Burdick & Jackson (U.S.A.). Water used in all experiments was from a Millipore Milli-Q system. Buffers and stock solutions of fluorescamine and 3-AMP were prepared as previously described.[2]

Procedures

Alkoxyamines were generated by photolysis of 1–1.5 mM solutions of the individual ketones or α-keto acids in the presence of ~800 μM 3-AMP in deaerated 0.2 M, pH 8.1 borate buffer. Upon irradiation, the ketones and α-keto acids undergo homolytic α-cleavage (Norrish type I reaction) to form carbon-centered radicals, which, in the presence of 3-AMP, are trapped as stable alkoxyamines.[1,2] To ensure that an adequate supply of the alkoxyamine products were obtained for the mass spectral measurements, samples were irradiated for 1 h at a light intensity of 245 mW/cm^2 employing the irradiation system described previously.[2] Following irradiation, samples (ca. 3 mL) were derivatized with 500 μL of the fluorescamine stock and then loaded onto a 0.2 × 5.5 cm enrichment column (EC), which was used in place of an injection loop. Samples were injected onto the HPLC by backflushing the EC and separated using the standard gradient elution (acetate buffer system) employed earlier.[2] The individual adducts were collected directly from the HPLC column (1.5–2.0 mL) and diluted to 10 mL with water. The lactone form of the adducts was extracted into 1 mL of chloroform,[6] which was subsequently washed three times with 10 mL of water. The volume of chloroform was reduced to ~50–250 μL by flushing with dry nitrogen.

A Finnigan 4510 quadrupole mass spectrometer, operating in the electron ionization mode at 100°C and at an electron energy of 50 eV, was employed. The analyzer was scanned from an m/z of 40 to 650 in 0.95 s. Approximately 1–5 μL of the chloroform solutions, corresponding to ~0.5 μg of compound, was spotted onto the gold tip of the direct insertion probe of the mass spectrometer. After solvent evaporation, the probe tip containing the sample was placed directly in the electron beam. The probe tip was then ramped from 50 to 350°C in 1 min. The samples desorbed as well shaped peaks spread over a few scans.

RESULTS AND DISCUSSION

Figure 1 provides a representative example of the mass spectra obtained for the radical adducts of fluorescamine-derivatized 3-AMP, while the inset (Figure 1) illustrates the general fragmentation pattern observed for these compounds. The relative ion abundances of these fragments are summarized in Table I for each of the compounds examined in this study. A prominent ion at m/z 290 was observed in all spectra. This ion most likely results from ionization at the N atom of the fluorescamine moiety followed by α-cleavage of the carbon–carbon bond linking the alkoxyamine (nitroxide) and fluorescamine substituents (Figure 1, inset). Because this ion was present in each spectrum and its formation was apparently unaffected by the fragmentation occurring at the alkoxyamine moiety (Figure 1, vide infra), we have normalized all ion abundances relative to 100 for this fragment.

Except for VII, the spectrum for each compound showed the expected molecular ion. The absence of a molecular ion for VII is likely due to the low stability of the t-butyl radical adduct.[10,11] Generally, the molecular ion abundances decreased with

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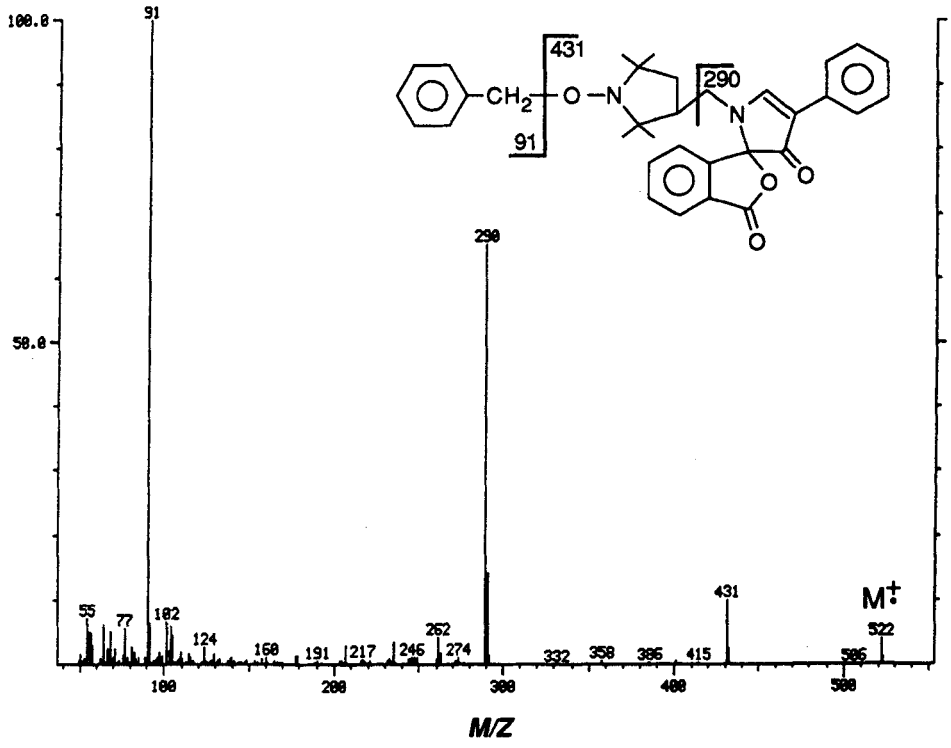

FIGURE 1 Mass spectrum of the benzyl radical adduct of 3-AMP-fluorescamine (Comp. IV, Table I). The inset illustrates the general fragmentation pattern observed for this and other compounds examined in this study (see Table I).

increasing chain length or size of the adducts, apparently reflecting the relative stability of the individual alkoxyamines.

Clear evidence for fragmentation at the carbon–oxygen bond of the alkoxyamines was provided by the observation of ions corresponding to the adduct moieties and 3-AMP-fluorescamine (Figure 1, Table I). For compounds II–V, the presence of the ion at m/z 431 can be explained by a simple α-cleavage of the oxygen–carbon bond linking the adduct to the parent. However, for compounds VI–XI, a fragment at m/z 432 was observed in place of the 431 ion. This fragment may be produced by on-probe thermolysis of the alkoxyamines[10] followed by reduction of the parent prior to ionization or alternatively, by intermolecular reactions in the gas phase.[12] The relative abundance of this ion increased at higher sample loadings suggesting that intermolecular reactions are occurring either on the probe or in the gas phase. However, we cannot entirely exclude that this ion is produced through an intramolecular rearrangement.

For those adducts having sufficiently high mass, direct evidence for the structural identity of the trapped radical was provided by the high yield of the ion(s) corresponding to the adduct fragment(s). These fragments are most likely produced through α-cleavage of the oxygen–carbon bond of the alkoxyamine (vide supra) or through charged-site initiated cleavage(s) along the adduct backbone.

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TABLE I
Summary of mass spectral data

Precursor	Adduct	Compound	Ion masses and abundances (normalized to m/z 290 = 100)[a]			
			Adduct fragment(s)	Molecular ion	Parent fragment	Other ions
Parent[b]	–	I	[c]	431 (26)	[c]	416 (< 1); 401 (4)
Acetone	CH_3	II	[d]	446 (11)	431 (63)	415 (6)
3-Pentanone	CH_3CH_2	III	[d]	460 (26)	431 (4)	445 (92)[e]; 415 (4)
β-Phenylpyruvate	$C_6H_5CH_2$	IV	91 (154)	522 (6)	431 (15)	415 (< 1)
Phenylglyoxylate	$C_6H_5C(O)$	V	105 (509); 77 (72)	536 (82)	431 (2)	416 (10); 401 (70)
3-Methyl-2-Butanone	$(CH_3)_2CH$	VI	43 (334)	474 (12)	432 (55)	417 (57); 401 (5)
3,3-Dimethyl-2-Butanone	$(CH_3)_3C$	VII	57 (530)	488 (0)	432 (125)	417 (78); 401 (9)
Pyruvate	$CH_3C(O)$	VIII	43 (226)	474 (12)	432 (226)	417 (93)
3-Pentanone	$CH_3CH_2C(O)$	IX	57 (335)	488 (19)	432 (227)	417 (64); 401 (13)
α-Ketovalerate	$CH_3(CH_2)_2C(O)$	X	71 (260); 43 (271)	502 (9)	432 (215)	417 (65); 401 (39)
α-Ketocaproate	$CH_3(CH_2)_3C(O)$	XI	85 (197); 57 (195); 43 (45)	516 (8)	432 (261)	417 (55); 401 (20)

[a] Values in parentheses are ion abundance normalized to m/z 290 = 100.
[b] 3-AMP-fluorescamine
[c] Not applicable
[d] Below observable mass range
[e] 445 (M–15) corresponds to loss of methyl group from adduct

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A limited number of other ions were seen at high mass. While we could postulate pathways for the production of these ions, advanced MS and/or labelling experiments would be required for unequivocal assignments. Thus, we have simply listed these ions and their abundances for completeness (Table I).

This study has shown that electron impact ionization of the radical adducts of 3-AMP-fluorescamine produces relatively simple and easily interpretable patterns of fragmentation. A significant advantage of this harsher ionization procedure is that one observes high yields of ions corresponding to the radical species originally trapped. Thus, direct information concerning the identity of these radicals can be obtained from the mass spectra.

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References

1. D.J. Kieber and N.V. Blough (1990) Fluorescence detection of carbon-centered radicals in aqueous solution. *Free Radical Research Communications*, **10**, 109–117.
2. D.J. Kieber and N.V. Blough (1990) Determination of carbon-centered radicals in aqueous solution by liquid chromatography with fluorescence detection. *Analytical Chemistry*, **62**, 2275–2283.
3. N.V. Blough and D.J. Simpson (1988) Chemically mediated fluorescence yield switching in nitroxide-fluorophore adducts: optical sensors of radical-redox reactions. *Journal of the American Chemical Society*, **110**, 1915–1917.
4. S.A. Green, D.J. Simpson, G. Zhou, P.S. Ho and N.V. Blough (1990) Intramolecular quenching of excited singlet states by stable nitroxy radicals. *Journal of the American Chemical Society*, **112**, 7337–7346.
5. J.L. Gerlock, P.J. Zacmanidis, D.R. Bauer, D.J. Simpson, N.V. Blough and I.T. Salmeen (1990) Fluorescence detection of free radicals by nitroxide scavenging. *Free Radical Research Communications*, **10**, 119–121.
6. E.G. Janzen, P.H. Krygsman, D.A. Lindsay and D.L. Haire (1990) Detection of alkyl, alkoxyl, and alkyperoxyl radicals from the thermolysis of azobis(isobutyronitrile) by ESR/spin trapping. Evidence for double spin adducts from liquid-phase chromatography and mass spectroscopy. *Journal of the American Chemical Society*, **112**, 8279–8284.
7. V.N. Bowry, J. Lusztyk and K.U. Ingold (1991) Calibration of a new horology of fast radical "clocks". Ring-opening rates for ring- and α-alkyl-substituted cyclopropylcarbinyl radicals and for the bicyclo[2.1.0]pent-2-yl radical. *Journal of the American Chemical Society*, **113**, 5687–5698.
8. M. Berger, J. Cadet and J. Ulrich (1985) Radiation-induced binding of 2,2,6,6-tetramethyl-1,4-piperidone-N-oxyl to thymidine in oxygen-free aqueous solutions. Isolation and characterization of the adducts. *Canadian Journal of Chemistry*, **63**, 6–14.
9. K.E. Murray and D.L. Ingles (1979) Identification of amines and amino acids as their fluorescamine derivatives by field desorption mass spectrometry. *Chemistry and Industry (London)*, **47**, 476–477.
10. D.W. Grattan, D.J. Carlsson, J.A. Howard and D.M. Miles (1979) The thermal decomposition of 1-(2′-cyano-2′-propoxy)-4-oxo-2,2,6,6-tetramethylpiperidine. *Canadian Journal of Chemistry*, **57**, 2834–2842.
11. J. Chateauneuf, J. Lusztyk and K.U. Ingold (1988) Absolute rate constants for the reactions of some carbon-centered radicals with 2,2,6,6-tetramethylpiperidine-N-oxyl. *Journal of Organic Chemistry*, **53**, 1629–1632.
12. F.W. McLafferty (1980) *Interpretation of Mass Spectra, 3rd Ed.* University Science Books, Mill Valley, Ca. **1980**, p. 218.

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